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PRINCIPAL INVESTIGATOR: Mark D. Noble, Ph.D.

CONTRACTING ORGANIZATION: University of Rochester  
Rochester, NY 14642

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## **INTRODUCTION**

This is the interim summary project report for Project 2 of grant W81XWH-08-1-0702. The goals of our work were to identify means of identifying cells, and individuals, that present with a more basal oxidized redox state and to identify molecular mechanisms that might functionally integrate such an oxidized state with observations that the multiple environmental insults that have been suggested to be involved in autism pathogenesis occur in many more children than those that develop ASD. This suggests that there is an underlying vulnerability phenotype that makes some children more vulnerable to such stressors. Taken together with our studies on the central importance of redox status in controlling cellular response to the environment, the observations that many children with ASD are more oxidized raises the possibility that this shift towards a more oxidative status provides a unifying principle that would explain such an increased vulnerability. The regulatory pathway that we have discovered to be central to understanding redox-based modulation of cell division, differentiation and survival clearly affects multiple cellular functions that appear to be related to the pathological changes seen in the CNS of children with autism. Moreover, the integration of these with the Nrf2 pathway provides molecular mechanisms that may not only provide better markers of a more oxidative state but would have the added value of explaining why the more oxidized cells of an individual with ASD do not simply reset themselves to create a normal redox balance. These discoveries provide new targets for resetting these metabolic problems, and continuation of this work looks likely to identify agents that could be used to address the oxidative abnormalities that look increasingly likely to be of relevance to the understanding of ASD pathogenesis. If we are correct that such redox abnormalities are responsible for the subtly different CNS development in children with ASDs, this will bring us to the point of being able to potentially recognize such children early and to provide protective interventions.

## **BODY**

The original aims of this project were as follows:

***Project 2, Aim 1: Analysis of the correlation between redox abnormalities in cells of the peripheral blood and cells of the developing brain***

- a) DoD regulatory review and approval of our UAMS IACUC-approved protocol (months 1-4)
- b) Optimization of methodologies for studying other metabolic aspects of redox balance (months 1-4; while we are awaiting DoD approval we will use established cell lines (that do not require regulatory approval) to optimize all analytic parameters relevant to the remaining components of Task 1)
- c) Analysis of the redox status (by dihydrocalcein fluorescence) of peripheral blood cells in multiple mouse strains at two different ages (Months 4-8)
- d) Analysis of the redox status of multiple CNS populations in multiple mouse strains (Months 3-12)
- e) Analysis of additional metabolic aspects of redox balance in cells of blood and developing brain (Months 3-12)
- f) Analysis of proteins that contribute to redox balance in the above cell populations (Months 12-18)
- g) Statistical Analysis and manuscript writing (Years 2 and 3)

***Aim 2: Analysis of the biological consequences of strain-dependent redox differences present in oligodendrocyte progenitor cells***

- a) Analysis of the relationship between redox state of oligodendrocyte progenitor cells isolated from different mouse strains and their ability to undergo division and differentiation in vitro (Months 6-18)
- b) Analysis of the correlates of redox status with the time course of myelination in vivo (Months 12-24)
- c) Analysis of the consequences of strain-associated differences in redox status for responsiveness of progenitor cells to thyroid hormone as an inducer of oligodendrocyte generation (Months 12-30)
- d) Analysis of the consequences of strain-associated differences in redox status for vulnerability of progenitor cells to physiological stressors of putative relevance to ASD pathogenesis (Months 12-30)
- e) Analysis of the role of activation of the redox/Fyn/c-Cbl pathway in 2a-d (Months 18-36)
- f) Statistical Analysis and manuscript writing: (Years 2 and 3)

We have made excellent progress in respect to our aims, and we also have multiple new pieces of this puzzle. Our attempts to understand how alterations associated with ASD might cause cells to exhibit a more oxidized redox status have been associated with good progress in our attempts to understand the underlying mechanisms by which cells might become more oxidized.

In summary, our work has provided the following outcomes:

In our work on this aim during Year 1 we first tried to address the problem that strain differences may themselves provide differences between cell populations that may or may not be relevant to redox status. To this end, we focused on goals 1e and 1f in order to first broaden the parameters that will be applied to the populations of 1b and 1c. By focusing on differences in basal redox state between oligodendrocyte progenitor cells (O-2A/OPCs) of the developing corpus callosum and cortex (Power et al., 2002), we were able to identify multiple physiological and protein expression parameters that are related to differences in redox status without concerns about whether differences were simply due to other effects of strain differences. This is an ideal situation in which to define redox-related differences in metabolite and protein expression without concern about differences that might be due to strain differences but unrelated to redox status. This work led to the identification of a wide range of differences of further potential

difference, including differences in levels of glutathione, in the ratio of ATP:ADP and the ratio of reduced:oxidized pyrimidine nucleotides, and differences in levels of bcl-2 and superoxide dismutase-1 and in the levels of  $\gamma$ -glutamyl-cysteinyl-synthase heavy chain ( $\gamma$ -GCS) (Fig. 1B, C), the rate-limiting enzyme in glutathione biosynthesis (Deneke and Fanburg, 1989).

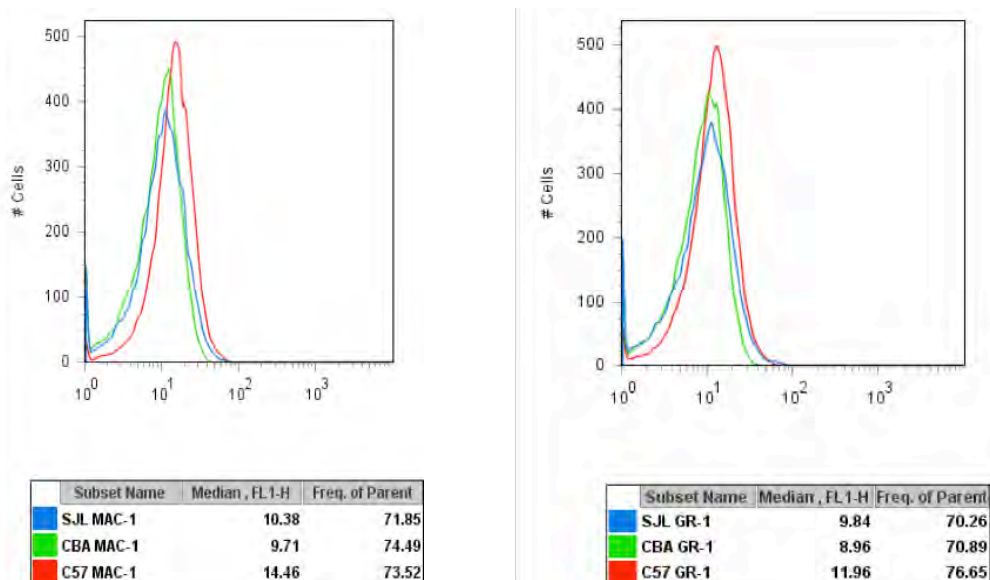
As per our original research plan, we now been applying our analyses to the lymphoid system. The primary goal in this part of Project 2 was to determine whether analysis of lymphoid and myeloid populations, or any other readily obtainable cell populations, would provide information on redox state that mirrored the redox state found in the progenitor cells of the developing CNS. As it is not possible to obtain CNS cells from children with autism, it is critical to determine whether other cell populations can be used to obtain information on basal redox status.

Analysis of multiple cell populations from the bloodstream has now revealed that both B lymphocyte and T lymphocyte populations show the same differences in redox status as we previously detected in oligodendrocyte progenitor cells. In contrast, these differences are not as reliably detected in myeloid populations, such as macrophages and neutrophils.

In these experiments, cells were isolated from the bloodstream of six-month old mice (using an orbital blood draw) and analyzed by flow cytometry using a combination of directly conjugated cell type-specific antibodies and a particular dye and we have found to be optimally useful in analysis of intracellular redox state. This dye, dihydro-CalceinAM, fluoresces when oxidized; thus cells that are more brightly labeled show a more oxidized metabolic status. The antibodies used to identify specific cell types were standard in literature, with the Mac1 antibody used to identify macrophages, GR-1 to identify neutrophils, B220 to identify B cells and CD3 to identify T cells.

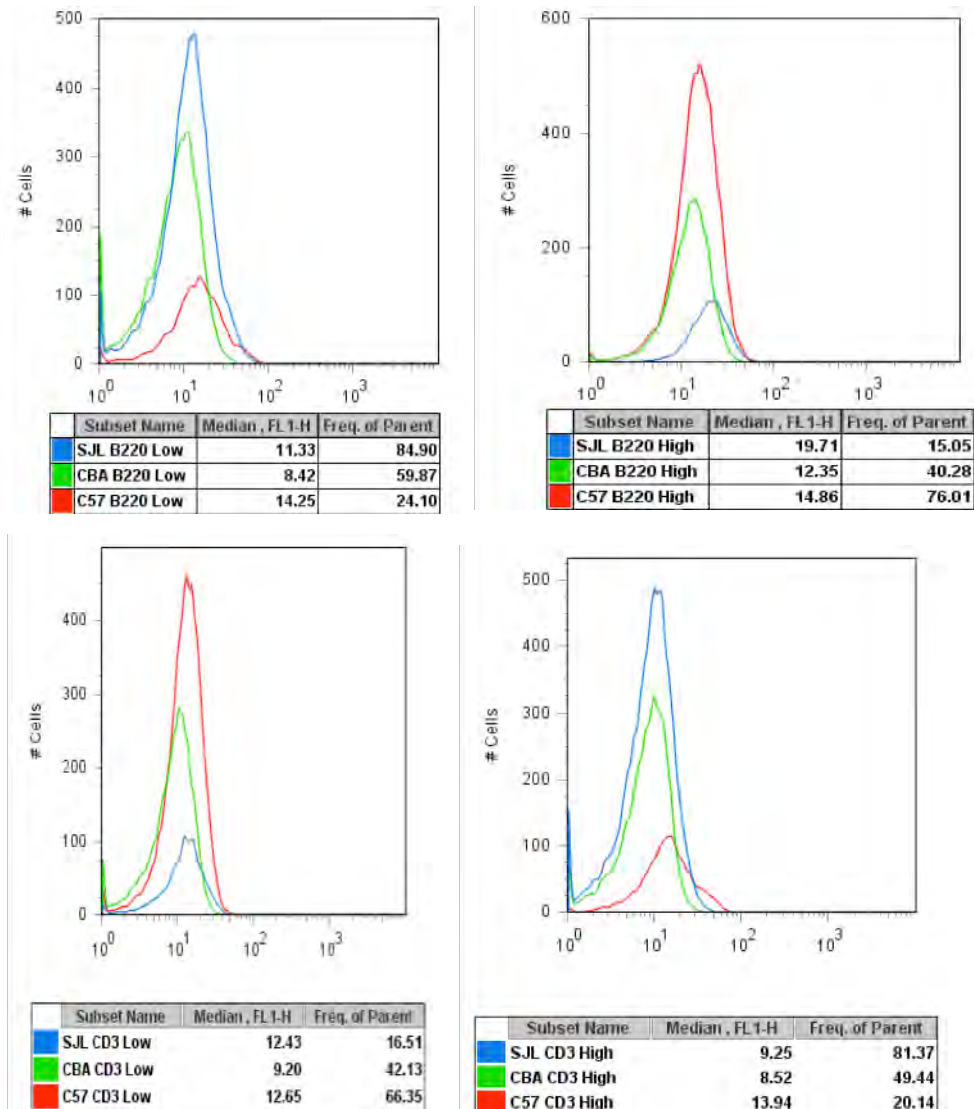
As shown in Figure 1, there was a trend of correlation between redox status of macrophages or neutrophils with our previous observations of the redox status of oligodendrocyte progenitor cells. We previously found that such cells were more oxidized in the developing brains of SJL and C57/BL6 mice than in the brains of identically aged CBA mice. In the myeloid populations, it was the case that cells derived from C57/BL6 mice were more oxidized than those derived from CBA mice. The labeling of cells derived from SJL mice with DHC did not give significantly different redox outcomes from those of CBA mice, however.

Figure 1. Analysis of intracellular redox status with DHC fails to detect consistent differences in redox status of macrophages or neutrophils in different strains of mice known to have difference in redox status of oligodendrocyte progenitor cells. Cells were freshly isolated and labeled with Mac-1 or GR-1 antibodies to identify macrophages or neutrophils, respectively. Cells were colabeled with DHC to determine oxidative status.



When we examined T cell and B cell populations, we first observed that there was a bimodal distribution of staining intensity with the CD3 antibody (a marker of T cells) and also a bimodal distribution of staining intensity with the B220 antibody (a marker of B cells). We therefore analyzed each of these populations separately (identified as CD3-low and high, or B220-low or high).

**Figure 2.** Analysis of intracellular redox status with DHC reveals consistent differences in redox status of B cells and T cells in different strains of mice known to have difference in redox status of oligodendrocyte progenitor cells. Cells were freshly isolated and labeled with B220 or CD3 antibodies to identify B-cells or T-cells, respectively. Cells were colabeled with DHC to determine oxidative status. As B220+ cells and CD3+ cells both contained two distinct populations of cells with high or low levels of antigen expression, these were examined separately. As seen for T cells, analysis of the CD3-low population revealed a greater degree of difference for SJL vs CBA cells than see for the CD3-high populations.



<b>Redox</b>	<b>Low</b>			<b>High</b>		
	CBA	C57	SJL	CBA	C57	SJL
<b>B cells</b>	8.42	14.25	11.33	12.35	14.86	19.71
<b>T cells</b>	9.20	12.65	12.43	8.52	13.94	9.25

**Table 1.** Summary of analysis of B cell and T cell populations with DHC. The numbers in red indicate the most oxidized populations. As shown, with the exception of CD3-high cells from SJL mice, the populations derived from SJL or C57BL/6 mice are from 20%-69% more brightly labeled (i.e., more oxidized) than comparable antigenically defined populations isolated from CBA mice.

We found that the redox status of T cells and B cells correlated well with our previous observations on the redox status of oligodendrocyte progenitor cells, regardless of the intensity of staining with either CD3 or B220 antibody. As shown in Figure 2, CD3+ T cells derived from either SJL or CBA mice were consistently more oxidized than those derived from CBA mice, with the sole exception to this observation being the CD3-high T cell populations examined in SJL mice. Here, as for macrophages and neutrophils, although there was a slightly greater DHC fluorescence detected in the SJL-derived cells than in the CBA-derived cells, but the difference was considerably smaller than that observed in the other lymphocyte populations analyzed. These data are shown in Figure 2 and summarized in Table 1.

The results obtained in this part of the work were very encouraging in that they may provide a means of assessing basal metabolic status without requiring examination directly of cells of the CNS.

One of the primary goals of the second aim of this research was to determine whether having a more oxidized basal metabolic state alters development in a manner that causes any of the subtle changes in the CNS that resemble the differences observed in the brain of children with autism. Our primary focus has been on oligodendrocyte lineage development. As discussed, however, the findings and the core hypothesis of our research have offered the opportunity to make predictions about hippocampal development that, if true, would both further test our core hypothesis and would have important implications for the understanding of learning problems in children with autism spectrum disorders.

The work funded by this DOD award has led not only to greater confidence in our core hypothesis but to the finding that the core hypothesis of our work also offers important predictions for the development of other components of the CNS of great relevance to the understanding of autism spectrum disorders, and in particular to the analysis of dendrite formation in the hippocampus.

The molecular pathway that we have previously discovered as underlying the changes in the cellular function caused by oxidation is one that converts small increases in oxidative status into accelerated degradation of specific receptor tyrosine kinases (RTKs) that are critical in such cellular functions as cell division and cell survival (Li et al., 2007). In this pathway, increased oxidative status activates Fyn kinase, leading in turn to activation of its target, the c-Cbl ubiquitin ligase. This enzyme then attaches ubiquitin to its target proteins, leading to their accelerated degradation.

One of the target receptors of the redox/Fyn/c-Cbl pathway is the c-Met receptor, a protein that plays an important role in many biological processes but is of particular interest in the context of autism. The connection between the c-Met receptor and autism is twofold. First, a polymorphism in the promoter region of the gene encoding this receptor has been observed at high frequency in autism. Moreover, examination of the brains of individuals carrying this polymorphism demonstrates a reduced level of the c-Met protein in their CNS (Campbell et al., 2006; Campbell et al., 2007). As activation of the redox/Fyn/c-Cbl pathway also leads to reductions in levels of the c-Met protein, such activation represents another means of achieving the lower protein levels that are of interest in regards to autism. The second reason for being particularly interested in either mutations or regulatory pathways that cause a reduction in the level of the c-Met protein has to do with the function of this protein in modulating dendrite generation in neurons of the hippocampus. Work by other researchers has revealed that stimulation of this receptor promotes

dendrite generation (Lim and Walikonis, 2008). This is potentially of great relevance to autism because the hippocampus is central to learning, and alterations in learning ability are of considerable concern in a large number of children diagnosed with autism spectrum disorders. Moreover, it has been previously reported that there is a reduced complexity of dendritic structure in the hippocampus of children with autism (Raymond et al., 1996).

When we analyzed the levels of the c-Met protein in the hippocampus of SJL mice, we found that these protein levels were much reduced compared with that found in the hippocampus of CBA mice (not shown). This is precisely as predicted by the redox/Fyn/c-Cbl hypothesis, as SJL mice are intrinsically more oxidized than CBA mice.

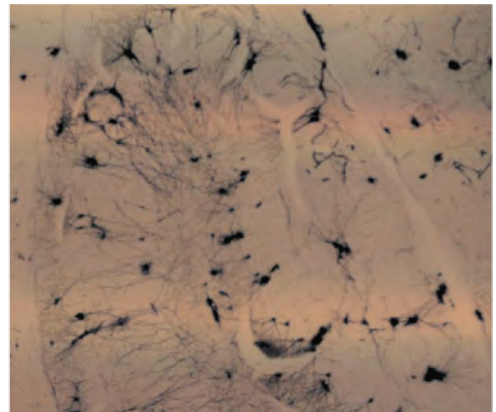
If the current understanding of the role of the c-Met protein is correct then a reduction in levels of this protein in the developing hippocampus should lead to a reduction in dendrite formation. Such an observation would provide a potentially direct linkage between oxidative abnormalities and a disruption in normal neuronal development.

We therefore analyzed dendritic complexity in the hippocampus of SJL and CBA mice. We focused our attention on animals that were 15 days old, as development during this period corresponds with that seen in the human CNS during the first 6 to 12 months of life. This analysis was conducted by using Golgi staining to reveal neuronal architecture, as illustrated in **Figure 3**. After performing the Golgi-staining, sections were imaged on a spinning disk confocal microscope. *Neurolucida*<sup>TM</sup> software was used to trace the hippocampal neurons. Once the cell body, axons and dendrites were traced the information was uploaded into *NeuroExplorer*<sup>TM</sup> for analysis of the following parameters:

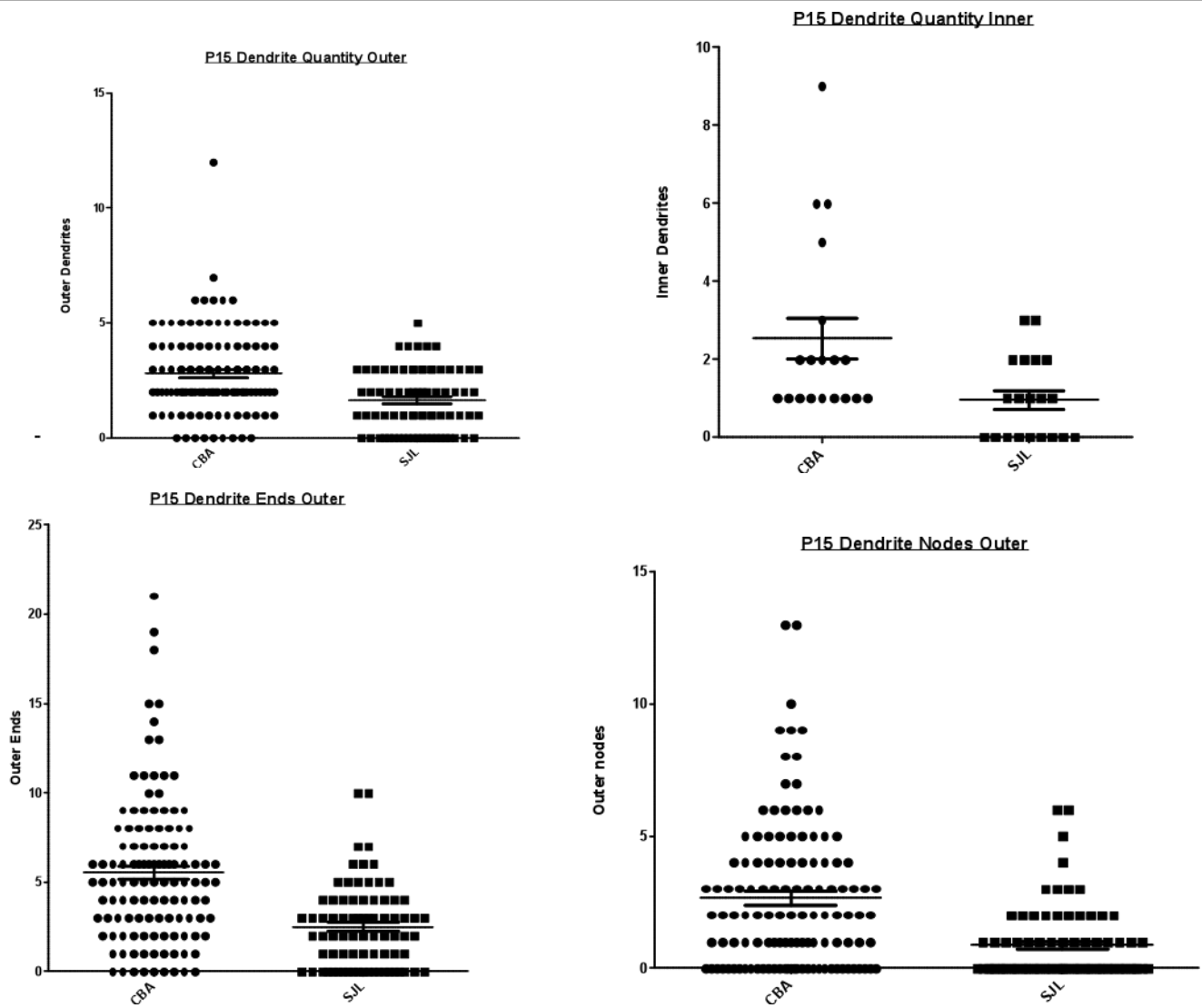
- *Quantity of dendrites.*
- *Number of dendrite nodes.*
- *Number of dendrite ends.*

In order to distinguish between different regions of the hippocampus, neurons were separately analyzed for those in the “outer” region of the hippocampus, closer to the cortex.

These analyses revealed several striking differences between hippocampus dendritogenesis in CBA vs SJL mice. Dendritic complexity in SJL mice was markedly reduced as compared with CBA mice of the same age. As shown in Figure 4, the quantity of dendrites was markedly lower in the hippocampi of SJL mice. This was reflected in a grouping of the dendritic numbers towards the lower number of values, as compared with the broader distribution of values for CBA mice. Similar outcomes were obtained for analysis of dendritic nodes, which were more numerous in the hippocampus of CBA mice than in SJL mice. As predicted from such outcomes, the number of dendritic ends was also lower in SJL mice (data not shown). Examples of the data from these studies are shown in Figure 4 and summarized in Table 2. Numerical values in Table 4 are provided with preliminary probability values as this analysis is ongoing. Nonetheless, even for the samples taken thus far, the probability that the differences seen is significant is high for all parameters examined.



**Figure 3.** Golgi staining of the hippocampus of a P15 mouse reveals detailed neuronal architecture. Detailed quantitative analysis of images such as these provides the data presented in Figure 4 and Table 2.



**Figure 4.** Analysis of dendritic complexity in SJL and CBA mice reveals reduced complexity in the hippocampus of P15 SJL mice as compared with that seen in CBA mice. Neurons were separately analyzed according to their location in the hippocampus. As seen, the number of dendrites and the number of nodes clusters at lower values for neurons in the hippocampus of SJL mice.

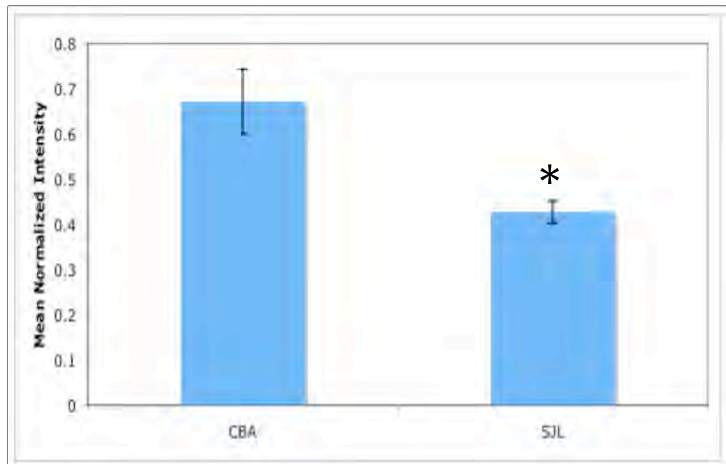
Neuronal parameter analyzed	CBA <i>n</i>	CBA mean	SJL <i>n</i>	SJL mean	Preliminary <i>p</i> values
Dendrite nodes outer	119	2.65	78	0.87	<.0001
Dendrite nodes inner	19	1.21	20	0.25	0.0368
Dendrite ends outer	119	5.52	78	2.49	<.0001
Dendrite ends inner	19	3.79	20	1.2	0.0081
Dendrite quantity outer	119	2.8	78	1.63	<.0001
Dendrite quantity inner	19	2.53	20	0.95	0.0084

**Table 2.** Summary of findings to date on analysis of dendritic complexity in the hippocampus of P15 SJL and CBA mice. The value for *n* is the number of neurons analyzed in their entirety. For each strain, neurons from a minimum of 3 different mice were examined. Standard deviations are not provided for this analysis as it is still ongoing, and *p* values should be considered as preliminary. Nonetheless, it is clear that the predicted outcome of less dendritic complexity in the developing hippocampus of more oxidized SJL mice, which have lower c-Met levels, looks likely to be correct.

*Additional information relevant to Project 2*

Observations discussed thus far support the following inter-related hypotheses:

- The redox state of B cells and CD3-low T cells is predictive of the redox state of cells of the CNS.
- Cells that are more oxidized will show increased activation of the redox/Fyn/c-Cbl pathway.
- Activation of the c-Cbl pathway leads to accelerated degradation of receptor tyrosine kinases that are targets of c-Cbl.



**Figure 5.** Examination of EGFR levels in skeletal muscle of CBA and SJL reveals significantly lower levels in muscle isolated from SJL mice. This outcome is as predicted if the analysis of lymphocytes and oligodendrocyte progenitor cells is indicative of redox status in other tissues of the body, and such increased oxidation leads to higher basal levels of activation of the redox/Fyn/c-Cbl pathway.

To begin to define the boundaries of the above hypotheses, we asked whether the information obtained through these studies is predictive of outcomes in other tissues of the body. To this end, we examined the levels of the receptor for epidermal growth factor (EGFR), another target of the c-Cbl ubiquitin ligase. As shown in our preliminary analysis (**Figure 5**), the levels of EGFR in skeletal muscle of SJL mice are significantly lower than in the skeletal muscle of CBA mice, precisely as predicted by the redox/Fyn/c-Cbl hypothesis.

All of the above findings converge on clear demonstration that redox states can be very different in differing strains of mice and that such redox differences have predictable consequences in respect to outcomes (c-Met expression and dendritic complexity) that are directly relevant to the understanding of ASD.

There is a clear question that follows from the work thus far, and it is this question that became integrated into our work in Year 3 and in the no-cost-extension that followed. The goal of Aim 1(f) is to identify proteins that contribute to the redox balance in the different cell populations of interest. This work started with a list of candidate proteins with the knowledge that further data would lead to expansion of this list. We also noted in our proposal that oxidation caused by disrupted function of the *ataxia telangiectasia mutated* gene caused changes in multiple proteins that were abnormally regulated in this condition, several of which had been shown to be also altered in children with ASD and noted that we would also consider these proteins, the goal being to come up with a way of defining a cell as being more oxidized at a level that would provide functional information relevant to the goals of Aim 2.

The question underlining our research in Aim 2 is whether it is possible to provide a mechanistic hypothesis to explain the observations that the multiple environmental insults that have been suggested to be involved in autism pathogenesis occur in many more children than those that develop ASD. This suggests that there is an underlying vulnerability phenotype that makes some

children more vulnerable to such stressors. Taken together with our studies on the central importance of redox status in controlling cellular response to the environment, the observations that many children with ASD are more oxidized raises the possibility that this shift towards a more oxidative status provides a unifying principle that would explain such an increased vulnerability. Our molecular analyses of the mechanisms by which redox status controls the function of cells critical in development of the central nervous system (CNS) further suggests that it is now possible to identify the reasons for this increased vulnerability at the level of detailed analysis of signaling pathway function. Such a possibility would provide rational strategies for developing means of altering vulnerability. Moreover, the regulatory pathway that we have discovered to be central to understanding redox-based modulation of cell division, differentiation and survival clearly affects multiple cellular functions that appear to be related to the pathological changes seen in the CNS of children with autism.

In other words, the combined goals of Aims 1 and 2 were to provide an understanding of how differences in redox state can be recognized, what their effects are on cell function and how these processes were regulated at the level of molecular mechanism.

To integrate the descriptive analysis of Aim 1 with the goals of Aim 2 it is critical to identify molecular mechanisms that underlie the differences in redox state between different cell populations – and happily it turns out that key clues were present in the data provided in our original proposal and our Year 1 progress report. We just needed a particular discovery from one of our parallel areas of research to place these clues into the right perspective and teach us how to integrate all of the aims of this project together into a seamless mechanistic context. This is quite exciting, as a mechanistic integration of Aims 1 and 2 holds hope of providing new therapeutic strategies.

### *Why do cells have different redox states?*

In the second year of our work we showed that pharmacological manipulation of cells to change redox state was sufficient to alter vulnerability to physiological stressors relevant to the CNS inflammation that has been reported to exist in ASD – but this can only be seen as part of the story. Why do different cells have different redox states? What pathway is in control? The data presented in our original proposal and in our Year 1 project report contained important clues to the answer to this problem, but we needed discoveries made in parallel research projects to teach us how to interpret this data in a useful manner.

Several of the indicators of redox state that we study are regulated by a single pathway called the anti-oxidant response element (ARE) pathway, which itself is regulated by the Nrf2 transcription factor. Oxidation has the potential to have such a plethora of negative consequences, that cells have to possess means of restoring a more appropriate redox balance. This is indeed the case, and evolution has provided entire pathways dedicated to this, of which the most important one seems to be the Nrf2/ARE pathway. Nrf 2 is activated by oxidation and causes expression of a wide range of enzymes that have the consequence of making cells more reduced. Moreover, Nrf2-mediated regulation of Phase II detoxifying enzymes provides the central means by which cells respond to toxic insults. Glutathione content is regulated in large part by the Nrf2 pathway, and multiple enzymes related to glutathione biosynthesis are controlled by this pathway.

The second major contributor to cellular redox state that is relevant to ASD is mitochondrial function. Abnormal mitochondrial function is of increasing interest as a risk factor for ASD.

Both the Nrf2 pathway and mitochondrial function are relevant to the indicators of redox state specified in our original proposal. For example, in Year 1 we showed that oligodendrocyte progenitor cells (O-2A/OPCs) that are isolated from the cortex of young rats, and that are more oxidized because of developmental differences in their redox regulation, have high levels of bcl-2, gamma-glutamyl-cysteinyl synthetase (g-GCS, the rate limiting enzyme in glutathione synthesis) and glutathione. In contrast, O-2A/OPCs isolated from the corpus callosum of the same animals have an intrinsically more oxidized basal redox status than do the cortical progenitors, and have lower levels of bcl-2, g-GCS and reduced glutathione. These observations are of particular interest as bcl-2 controls mitochondrial function by blocking mitochondrial permeability transition pore and thus helps to keep cells more reduced.

It is also important to stress that by examining the differences in redox regulation in different cell populations isolated from the same animals we are able to focus attention on differences in redox status that are due to epigenetic modification of gene expression. The hypothesis that there is epigenetic regulation of redox balance is central to the hypotheses of Project 1. Because our experimental observations are not influenced by genetic differences between cells from different strains of mice, the outcomes can only be due to epigenetic differences in regulation of redox balance – and perhaps also of the ability to respond to oxidative stress.

*Nrf2 pathway dysfunction may be at the heart of understanding the reasons why some oxidized cells fail to reset their normal redox balance.* In parallel work (funded by other resources) we also have discovered, through examination of another disease (ataxia telangiectasia) in which cells are chronically oxidized, that one mechanism of disease appears to be Nrf2 dysfunction and inability to restore a normal redox balance.

The observations that alterations in Nrf2 function could be central to understanding differences in redox state, combined with our findings on increased bcl-2 expression in cells that were more reduced, provided the clues that were needed to integrate our different observations into a single hypothesis that yields a series of testable predictions.

At its current stage of development, the hypothesis that integrates many of the components of this research effort into a single pathway, stretching from changes observed at the descriptive level to the mechanisms responsible for these changes, can be stated as follows: Epigenetic changes in Nrf2 activity are responsible for fundamentally different basal redox states and in the ability to respond to agents that perturb redox state. When Nrf2 activity is set at a lower level, cells are intrinsically more oxidized and more susceptible to agents that further increase oxidative status. These changes cause activation of the redox/Fyn/c-Cbl pathway, leading to increased degradation of important receptor tyrosine kinases (RTKs) at the cell surface. This leads to decreases in NF-kB activity, which in turn leads to reduced levels of bcl-2 and also of g-GCS. Reductions in levels of bcl-2 further work to keep cells more oxidized by allowing greater efflux of reactive oxidative species from mitochondrial permeability transition pores. Thus, cells with reduced Nrf2 activity are maintained in a chronically oxidized state that is responsible for increased activity of the redox/Fyn/c-Cbl pathway, and activation of the redox/Fyn/c-Cbl pathway reduces NF-kB activity and levels of bcl-2 and of g-GCS, thus helping to maintain the chronically oxidized phenotype.

This hypothesis is summarized diagrammatically in the following two figures.

**The case of cells that have a more reduced basal redox state can be represented as follows:**

In contrast, the case of cells that have a more oxidized basal redox state can be represented

## **Intrinsically reduced state**

### *Markers and Mechanisms*

### *Implications*

*Increased* Nrf2-mediated activation of ARE-mediated gene transcription

Predicts markers of redox balance that serve as useful markers and as critical regulators of the ability to respond to oxidative stressors and physiological insults  
Examples: glutathione reductase, X<sub>c</sub>T cystine transporter, *increase* of reduced over oxidized glutathione

*Reduced* activation of redox/Fyn/c-Cbl pathway

*Higher* levels of tyrosine kinase receptors and other proteins that are of critical importance modulating cell division, differentiation and survival  
Examples: *higher* levels of c-Met (mutations of which are risk factors for ASD), *higher* levels of NF-kB activity (which leads to *higher* levels of bcl-2 and gamma-glutamylcysteine

*Increased* levels of bcl-2 and Gamma-glutamylcysteine synthetase

*Decrease* in efflux of reactive oxidative species from mitochondria, thus keeping cells more *reduced*; protection against physiological stressors, and particularly those that cause mitochondrial pore opening; *increased* glutathione levels, thus *increasing* protection against physiological stressors (so long as levels of glutathione reductase (which is Nrf2 regulated) are *high*)

as follows:

## **Intrinsically oxidized state**

### *Markers and Mechanisms*

### *Implications*

*Decreased* Nrf2-mediated activation of ARE-mediated gene transcription

Predicts markers of redox balance that serve as useful markers and as critical regulators of the ability to respond to oxidative stressors and physiological insults  
Examples: glutathione reductase, X<sub>c</sub>T cystine transporter, *decrease* of reduced over oxidized glutathione

*Increased* activation of redox/Fyn/c-Cbl

*Lower* levels of tyrosine kinase receptors and other proteins that are of critical importance modulating cell division, differentiation and survival  
Examples: *lower* levels of c-Met (mutations of which are risk factors for ASD), *lower* levels of NF-kB activity (which leads to *lower* levels of bcl-2 and gamma-glutamylcysteine synthetase)

*Decreased* levels of bcl-2 and Gamma-glutamylcysteine

*Increase* in efflux of reactive oxidative species from mitochondria, thus keeping cells more *oxidized*; less protection against physiological stressors, and particularly those that cause mitochondrial pore opening; *decreased* glutathione levels, thus *decreasing* protection against physiological stressors (so long as levels of glutathione reductase (which is Nrf2 regulated) are *low*)

This hypothesis offers a large number of predictions that seemed directly relevant to the goals of this research effort. Among the predictions that we have tested thus far are the following:

- (i) Cells that are more oxidized for epigenetic reasons (i.e., are not due to strain/genetic differences) will show less ARE activity than cells that are more reduced for epigenetic reasons.
- (ii) Activation of Nrf2 will cause oxidized cells to become more reduced, including having higher levels of glutathione, and to alter their biological properties to now behave like cells that are more reduced for epigenetic reasons.
- (iii) Inhibition of Nrf2 will cause reduced cells to become more oxidized, including having lower levels of glutathione, and to alter their biological properties to now behave like cells that are more oxidized for epigenetic reasons.
- (iv) Elevation of levels of bcl-2 will reduce the ability of pro-oxidants to activate the redox/Fyn/c-Cbl pathway.
- (v) Pro-oxidant inducers of differentiation work through activation of the redox/Fyn/c-Cbl pathway, and can be blocked by increased activation of Nrf2 or by increased expression of bcl-2.

We have tested all of these predictions, and all of them appear to be correct. Specifically,

- (i) O-2A/OPCs isolated from the corpus callosum (and which are intrinsically more oxidized) show lower levels of ARE activity than progenitor cells isolated for the cortex (and which intrinsically are more reduced).
- (ii) Increasing the levels of Nrf2 activity by overexpressing Nrf2 in corpus callosum O-2A/OPCs makes these cells more reduced and reduces the tendency of these cells to undergo differentiation into oligodendrocytes.
- (iii) Inhibiting Nrf2 activity by expression of one of the inhibitory binding partners Keap1 (which keeps Nrf2 from entering the nucleus and thus prevents Nrf2-mediated activation of the ARE promoter) makes O-2A/OPCs more oxidized and increases the early spontaneous generation of oligodendrocytes.
- (iv) Elevation of levels of bcl-2 inhibits the ability of the pro-oxidant thyroid hormone (thought to be the major physiological regulator of oligodendrocyte differentiation) to induce O-2A/OPCs to become oligodendrocytes.
- (v) Increasing Nrf2 activity (by overexpression of the Nrf2 protein) or increasing levels of bcl-2 both decrease the ability of thyroid hormone to activate the redox/Fyn/c-Cbl pathway and induce differentiation of dividing O-2A/OPCs into oligodendrocytes.

#### *The work of the no-cost extension*

The above problems are precisely the problems that are at the core of the goal of this grant, which is to improve our understanding of oxidative abnormalities in ASD. Our work has revealed a mechanism that ties increased oxidative status together with problems ranging from increased vulnerability to physiological stressors (including environmental toxicants) to reduced levels of a receptor (c-Met) mutated in ASDs to reduced dendritic complexity in the hippocampus. We now may be at the cusp of integrating this research with the identification of molecular mechanisms that would not only provide better markers of a more oxidative state (our Aim 1) but would have the added value of explaining why the more oxidized cells of an individual with ASD do not simply reset themselves to create a normal redox balance. This would provide new targets for resetting these metabolic problems, as there are some very interesting Nrf2 regulators (with potential clinical application) that we will examine in this regard. We believe that there is a very good chance that this work is going to identify agents

that could be used to address the oxidative abnormalities that look increasingly likely to be of relevance to the understanding of ASD pathogenesis. If we are correct, this would have profound implications for understanding how to reset the redox balance in individuals with ASDs.

In our no-cost extension we proposed to carefully use the remaining resources in this research program to conduct the following experiments:

- (i) Test the prediction that cells that are intrinsically more oxidized for developmental (epigenetic) reasons also show reduced levels of the ARE-regulated proteins glutathione reductase (which is critical for maintaining glutathione in a reduced state) and the xCT cystine transporter (which is a critical cystine-uptake mechanism for glutathione synthesis during oxidative stress). This will be done by analysis of both protein and mRNA levels for proteins of interest.
- (ii) Test the prediction that increasing Nrf2 activity in these more oxidized cells will reset the redox balance to a more reduced status and will thus lead to reduced activation of the redox/Fyn/c-Cbl pathway, increased NF-kB activity, increased bcl-2 and g-GCS levels and increased resistance to physiological stressors thought to be of relevance in ASD. This will be done by genetic interference with expression of Nrf2 and also by treatment with pharmacological activators of Nrf2.
- (iii) Test the prediction that another way to favorably alter the redox balance in cells that are intrinsically more oxidized will be to suppress c-Cbl function, leading to increased bcl-2 and g-GCS levels (due to increased NF-kB activity), a more reduced redox status and increased resistance to physiological stressors thought to be of relevance in ASD. This will be done by genetic suppression of c-Cbl function.
- (iv) Test the prediction that our findings on the epigenetic regulation of intrinsic redox balance (i.e, in cells that are from the same animals and thus are genetically identical) provide improved means of analyzing redox balance in animals that have intrinsically different redox states due to genetic reasons (in our case, due to strain differences in redox balance). This will be carried out by analyzing ARE activity, levels of glutathione reductase and the xCT transporter (targets of ARE-mediated transcriptional regulation) levels of bcl-2 and g-GCS (targets of NF-kB mediated transcriptional regulation) and ratios and absolute amounts of reduced:oxidized glutathione. The prediction is that more oxidized cells will show lower levels of glutathione reductase, xCT transporter, bcl-2, g-GCS and a decrease in the ratio of reduced:oxidized glutathione. These experiments will be conducted on O-2A/OPCs and on B and T lymphocytes from strains of animals that our prior work in this project (original proposal and Year 2 progress report) were shown to have different redox states for genetic reasons.
- (v) Test the prediction that both in cells that are more oxidized for epigenetic or for genetic reasons, increasing Nrf2 activity or decreasing c-Cbl activity will provide protection against physiological stressors thought to be of relevance to ASD (e.g., environmental toxicants, inflammatory mediators). Cells will be manipulated by both genetic and pharmacological means (in the latter case, thus far, just for the Nrf2 pathway as pharmacological modifiers of c-Cbl activity have not yet been identified). These experiments will be conducted on O-2A/OPCs.

We have made excellent progress on goals (i)-(iii) above. In respect to goal (iv) ongoing research is revealing more complex regulation of these components than the literature would lead

one to believe. This work is ongoing and will become part of our further grant applications. Work on goal (v) has been initiated and looks quite promising.

### **Key Research Accomplishments**

- We have identified a wide range of redox-related factors that can be used to distinguish cells with different basal redox states, including differences in levels of glutathione, in the ratio of ATP:ADP and the ratio of reduced:oxidized pyrimidine nucleotides, and differences in levels of bcl-2 and superoxide dismutase-1 and in the levels of  $\gamma$ -glutamyl-cysteinyl-synthase heavy chain ( $\gamma$ -GCS), the rate-limiting enzyme in glutathione biosynthesis.
- Analysis of multiple cell populations from the bloodstream shows that both B lymphocyte and T lymphocyte populations show the same differences in redox status as detected in oligodendrocyte progenitor cells. In contrast, these differences are not as reliably detected in myeloid populations, such as macrophages and neutrophils.
- Analysis of the levels of the c-Met protein in the hippocampus of SJL mice (which are more oxidized) shows that these protein levels are much reduced compared with that found in the hippocampus of CBA mice (which are more reduced). This demonstrates the predictive value of our redox/Fyn/c-Cbl pathway analysis. As SJL mice are intrinsically more oxidized than CBA mice, our prediction was that this would lead to lower levels of c-Met, which is a c-Cbl target.
- We next tested the hypothesis that SJL mice would have impaired neuronal dendrite generation, as has been observed in autism. This was our prediction due to the role of c-Met in dendritic regulation. Our analyses revealed several striking differences between hippocampus dendritogenesis in CBA vs SJL mice. Dendritic complexity in SJL mice was markedly reduced as compared with CBA mice of the same age. The quantity of dendrites was markedly lower in the hippocampi of SJL mice. This was reflected in a grouping of the dendritic numbers towards the lower number of values, as compared with the broader distribution of values for CBA mice. Similar outcomes were obtained for analysis of dendritic nodes, which were more numerous in the hippocampus of CBA mice than in SJL mice. As predicted from such outcomes, the number of dendritic ends was also lower in SJL mice.
- To integrate the descriptive analysis of Aim 1 with the goals of Aim 2 it was critical to identify molecular mechanisms that underlie the differences in redox state between different cell populations. The hypothesis that integrates many of the components of this research effort into a single pathway, stretching from changes observed at the descriptive level to the mechanisms responsible for these changes, can be stated as follows: Epigenetic changes in Nrf2 activity are responsible for fundamentally different basal redox states and in the ability to respond to agents that perturb redox state. When Nrf2 activity is set at a lower level, cells are intrinsically more oxidized and more susceptible to agents that further increase oxidative status. These changes cause activation of the redox/Fyn/c-Cbl pathway, leading to increased degradation of important receptor tyrosine kinases (RTKs) at the cell surface. This leads to decreases in NF-kB activity, which in turn leads to reduced levels of bcl-2 and also of  $\gamma$ -GCS. Reductions in levels of bcl-2 further work to keep cells more oxidized by allowing greater efflux of reactive oxidative species from mitochondrial permeability transition pores. Thus, cells with reduced Nrf2 activity are maintained in a chronically oxidized state that is responsible for increased activity of the redox/Fyn/c-Cbl pathway, and activation of the redox/Fyn/c-Cbl pathway reduces NF-kB

activity and levels of bcl-2 and of g-GCS, thus helping to maintain the chronically oxidized phenotype.

- Testing of multiple predictions of the redox Nrf2 hypothesis provides increasing strong support for this hypothesis. This hypothesis offers a large number of predictions that seemed directly relevant to the goals of this research effort. Specifically, we made the following predictions and observations:

- *Prediction:* Cells that are more oxidized for epigenetic reasons (i.e., are not due to strain/genetic differences) will show less ARE activity than cells that are more reduced for epigenetic reasons.

- *Outcome:* O-2A/OPCs isolated from the corpus callosum (and which are intrinsically more oxidized) show lower levels of ARE activity than progenitor cells isolated for the cortex (and which intrinsically are more reduced).

- *Prediction:* Activation of Nrf2 will cause oxidized cells to become more reduced, including having higher levels of glutathione, and to alter their biological properties to now behave like cells that are more reduced for epigenetic reasons.

- *Outcome:* Increasing the levels of Nrf2 activity by overexpressing Nrf2 in corpus callosum O-2A/OPCs makes these cells more reduced and reduces the tendency of these cells to undergo differentiation into oligodendrocytes.

- *Prediction:* Inhibition of Nrf2 will cause reduced cells to become more oxidized, including having lower levels of glutathione, and to alter their biological properties to now behave like cells that are more oxidized for epigenetic reasons.

- *Outcome:* Inhibiting Nrf2 activity by expression of one of the inhibitory binding partners Keap1 (which keeps Nrf2 from entering the nucleus and thus prevents Nrf2-mediated activation of the ARE promoter) makes O-2A/OPCs more oxidized and increases the early spontaneous generation of oligodendrocytes.

- *Prediction:* Elevation of levels of bcl-2 will reduce the ability of pro-oxidants to activate the redox/Fyn/c-Cbl pathway.

- *Outcome:* Elevation of levels of bcl-2 inhibits the ability of the pro-oxidant thyroid hormone (thought to be the major physiological regulator of oligodendrocyte differentiation) to induce O-2A/OPCs to become oligodendrocytes.

- *Prediction:* Pro-oxidant inducers of differentiation work through activation of the redox/Fyn/c-Cbl pathway, and can be blocked by increased activation of Nrf2 or by increased expression of bcl-2.

- *Outcome:* Increasing Nrf2 activity (by overexpression of the Nrf2 protein) or increasing levels of bcl-2 both decrease the ability of thyroid hormone to activate the redox/Fyn/c-Cbl pathway and induce differentiation of dividing O-2A/OPCs into oligodendrocytes.

## Reportable Outcomes

We are now working to integrate all findings together into a new understanding that will stretch from the identification of individuals with oxidative imbalances to implications of oxidative imbalance on the response to cellular stress to molecular mechanisms underlying such altered responses and on to mechanisms that may even explain the failure of oxidized cells (as are

present in individuals with ASDs) to repair their redox balance to a more physiologically normal level.

As there are sequential elements that need to be provided in order that this work will have the desired effect on the scientific community, our current plan is to publish this work in the following sequence:

Paper 1 (currently in preparation for submission) places the redox/Fyn/c-Cbl pathway at the center of understanding how the balance between self-renewing division and differentiation into a non-dividing terminally differentiated cells is regulated in oligodendrocyte progenitor cells. This paper also places mitochondrial regulation of reactive oxidative species production and release at the center of this regulation. This paper thus integrates redox regulation of progenitor cell function with other key cellular components, and provides the foundation needed to attract other researchers to this problem.

Paper 2 (draft text in progress) is focused on the regulation of the redox balance in progenitor cell populations that have cell-intrinsic regulation of this balance. As these progenitor cells are isolated from different regions of the developing CNS of the same animals there are no concerns about whether outcomes are due to strain differences. This paper will place the Nrf2 pathway in a primary regulatory position for normal development rather than simply as a response to oxidative stress. Such a developmental understanding of the function of Nrf2 is required for the next studies demonstrating what happens when this pathway does not function properly.

Paper 3 (draft text of progress) will provide the first paper on dysregulation of Nrf2 function in the context of human disease. Although this paper is focused on the disease ataxia telangiectasia, and the work has been conducted with funding from other sources, the experiments have often been informed by the knowledge gained from the DOD-sponsored work. Thus, it will be appropriate to recognize the DOD support in this paper.

Paper 4 (additional experiments being conducted) will report the effects of redox status on development of dendritic complexity.

## **Conclusions**

The goals of our work were to identify means of identifying cells, and individuals, that present with a more basal oxidized redox state and to identify molecular mechanisms that might functionally integrate such an oxidized state with observations that the multiple environmental insults that have been suggested to be involved in autism pathogenesis occur in many more children than those that develop ASD. This suggests that there is an underlying vulnerability phenotype that makes some children more vulnerable to such stressors. Taken together with our studies on the central importance of redox status in controlling cellular response to the environment, the observations that many children with ASD are more oxidized raises the possibility that this shift towards a more oxidative status provides a unifying principle that would explain such an increased vulnerability. Our molecular analyses of the mechanisms by which redox status controls the function of cells critical in development of the central nervous system (CNS) further suggests that it is now possible to identify the reasons for this increased vulnerability at the level of detailed analysis of signaling pathway function.

Such a possibility would provide rational strategies for developing means of altering vulnerability. Moreover, the regulatory pathway that we have discovered to be central to understanding redox-based modulation of cell division, differentiation and survival clearly affects multiple cellular functions that appear to be related to the pathological changes seen in the CNS of children with autism.

The integration of the above analyses with the Nrf2 pathway provides molecular mechanisms that may not only provide better markers of a more oxidative state (our Aim 1) but would have the added value of explaining why the more oxidized cells of an individual with ASD do not simply reset themselves to create a normal redox balance. This would provide new targets for resetting these metabolic problems, as there are some very interesting Nrf2 regulators (with potential clinical application) to be considered in this regard. We believe that there is a very good chance that the continuation of this work is going to identify agents that could be used to address the oxidative abnormalities that look increasingly likely to be of relevance to the understanding of ASD pathogenesis. If we are correct, this would have profound implications for understanding how to reset the redox balance in individuals with ASDs. If we are correct that such redox abnormalities are responsible for the subtly different CNS development in children with ASDs, this will bring us to the point of being able to potentially recognize such children early and to provide protective interventions.

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